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Adhesion of *Colletotrichum lindemuthianum* Spores to *Phaseolus vulgaris* Hypocotyls and to Polystyrene

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Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene was inhibited by the respiratory inhibitors sodium azide and antimycin A, indicating a requirement for metabolic activity in adhesion. Various commercial proteins and Tween 80 also reduced adhesion to both surfaces. Binding was enhanced by the presence of salts: sodium, potassium, calcium, and magnesium chlorides were equally effective. The removal of surface wax from hypocotyls by chloroform treatment greatly reduced their subsequent ability to bind spores. The results suggest a similar mechanism for spore adhesion to the plant surface and to polystyrene, involving purely physical surface properties rather than group-specific binding sites.

Adhesion of microorganisms to the surface of plant, animal, or inert material plays a crucial role in colonization by maintaining the organism in a favorable environment for growth, and in some cases adhesion may be a highly specific phenomenon (5). Many reports indicate that fungi can adhere to different surfaces (4, 5, 11, 17, 18, 20). However, the mechanisms involved have received little attention, in contrast to those of bacterial adhesion.

During infection of beans (*Phaseolus vulgaris* L.) by the fungal pathogen *Colletotrichum lindemuthianum*, the causal agent of anthracnose, the spore and appressorium adhere firmly to the plant surface (8). A mucilaginous sheath has been implicated in adhesion of the appressorium (8, 12), but the mechanism of spore adhesion is unknown. Adhesion of *C. lindemuthianum* spores to the plant surface may be a crucial factor in the spread of disease during spore dispersal, which is thought to be caused largely by splashing water during heavy rain (24). Another important function of adhesion may be to anchor the spore in an environment suitable for germination which is stimulated by substances from the plant (12) and in which physical contact of the germ tube with the plant surface can trigger appressorium formation (8, 13). In this paper the mechanism of adhesion is investigated in a comparative study of spore binding to *P. vulgaris* hypocotyls and to polystyrene.

MATERIALS AND METHODS

Spore suspensions. The methods used to culture *C. lindemuthianum*, race kappa, and to prepare spore suspensions have been described previously (25).

Plant material. Seeds of *P. vulgaris* cv. Simplobel, which is susceptible to race kappa, were obtained from Royal Sluis, Enkhuizen, The Netherlands. Seeds were germinated for 2 days between layers of moist filter paper in petri dishes at 26°C and then planted in vermiculite and grown for 7 or 8 days under fluorescent light (14 h of illumination per day) at 20°C.

Assay of spore adhesion to hypocotyls. The assay of spore adhesion to hypocotyls involved incubating hypocotyls in a spore suspension for an appropriate period and then counting the unbound spores with a Coulter Counter to obtain an estimate of the number of bound spores.

Hypocotyl segments, 2 cm long, were excised from immediately below the cotyledonary node of seedlings which were 2 to 3 cm high. The segments were briefly washed twice in distilled water to remove vermiculite particles which may interfere with spore counting. In the standard assay, 9×10^5 spores in either deionized water or 10 mM sodium citrate-phosphate buffer, pH 5.0, were added to 5-ml glass tubes. Deionized water or buffer containing the appropriate test substance was added to give a final volume of 3 ml. Two hypocotyl segments were then placed in each tube. Control mixtures, in which either spores or hypocotyls were omitted to provide counts of the total spores and of the background count due to contaminating particles from the hypocotyls, respectively, were included for all treatments. Tubes were sealed with plastic caps and rolled at 80 rpm at room temperature for the appropriate period on a roller mixer (Denley Technology, Sussex, England), and then the hypocotyls were removed. The spore suspensions were added to 20-ml test tubes. The assay tubes were then washed by adding 3 ml of phosphate-buffered saline (25 mM Sorenson phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.05% NaN_3) and mixing vigorously on a Vortex mixer. The washings were added to the spore suspensions, and the volume was made up to 13 ml by adding 7 ml of phosphate-buffered saline. The tubes were shaken vigorously by hand before the suspensions were counted in a model ZBI Coulter Counter (Coulter Electronics, Luton, England) with phosphate-buffered saline as the electrolyte at settings of 1/amplification = 1 and 1/aperture current = 1/4, with a window of 10 to 110. The counter was equipped with a 100 μm aperture and a 0.5-ml manometer. The matching switch was set at 20 kΩ. The background count for the controls without spores was subtracted from spore counts for the test mixtures to calculate the number of unbound spores. The number of bound spores was calculated as the difference between the number of unbound spores and that of the total spores. In some experiments surface wax was removed from hypocotyls before spore adhesion was assayed. Hypocotyls were dipped three times in $CHCl_3$ for 1 s per dipping (9) and then washed twice for 5 min in deionized water with gentle shaking. Untreated control hypocotyls were washed similarly.

Assay of spore adhesion to microtiter plate wells. A solution

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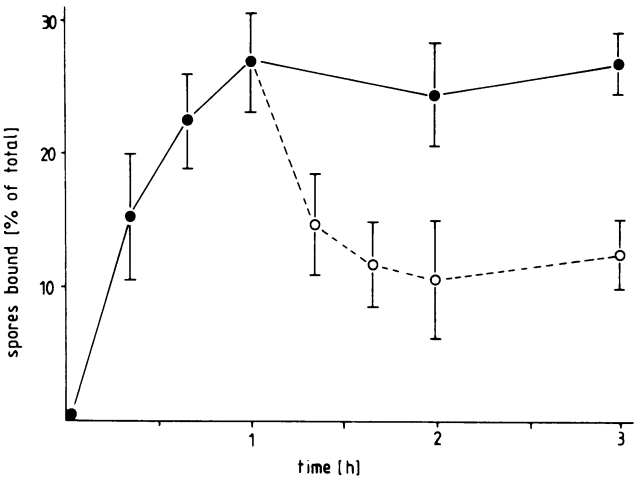

FIG. 1. Adhesion of *C. lindemuthianum* spores in deionized water to *P. vulgaris* hypocotyls and desorption as a function of time. Fivefold replication was used. Vertical bars indicate ±1 standard deviation. Symbols: ●, spore adhesion; ○, spore desorption. To examine spore desorption, hypocotyls were removed from the assay tubes after 1 h and transferred to fresh tubes containing 3 ml of deionized water. Mixing was continued, and the number of spores remaining bound was calculated from counts of the number of spores removed.

of the appropriate test substance (0.2 ml) in 10 mM citrate-phosphate buffer, pH 5.0, or deionized water was added to wells (7-mm diameter) of Nunclon Delta flat-bottomed polystyrene microtiter plates (Nunc, Roskilde, Denmark). A spore suspension (0.2 ml containing 2×10^6 spores per ml) in buffer or deionized water was then added. The well contents were mixed and left for 1 h to allow all the spores to settle onto the bottom of the wells. The total number of spores in an area 0.8 mm^2 at the center of each well was counted with the aid of a grid by examination from below with a Zeiss inverted microscope. The fluid from each well was removed by suction with a drawn-out Pasteur pipette held at the side of the well. Buffer or deionized water (0.4 ml) was added to the wells with an eight-channel dispenser (Dynatech Laboratories, Inc., Alexandria, Va.) to ensure uniform washing of the wells, and the fluid was removed. The washing procedure was repeated; then 50 μl of buffer or water was added carefully to each well to prevent the wells from drying out, and the spores still adhering at the centers of the wells were counted.

Chemicals. Cytochrome *c* (horse heart), hemoglobin (bovine type I), ovalbumin (grade V), bovine serum albumin (globulin-free), fetuin (type III), sodium poly-L-aspartate (molecular weight, 20,000 to 50,000), and antimycin A were from Sigma Chemical Co., St. Louis, Mo. Immunoglobulin (bovine, Cohn fraction II), protamine sulfate, and Tween 80 were from Serva, Heidelberg, Germany.

RESULTS

Adhesion to the plant surface. In the hypocotyl assay the total percentage of spores which bound reached a maximum after ca. 1 h of incubation and then remained essentially constant (Fig. 1). The average percentage of spores bound in different experiments ranged from 25 to 35 when the assay was performed in deionized water and from 35 to 50 when 10 mM citrate-phosphate buffer, pH 5.0, was used. When hypocotyls were transferred after 1 h of incubation to fresh tubes containing deionized water or buffer and the incubation was continued, more than half of the bound spores were removed (Fig. 1). Spores which were not bound after a 1-h incubation in the standard assay still showed some ability to bind to hypocotyls when these spores were subsequently tested, although the percentage of spores which bound was reduced by about half as compared with the first assay (data not shown). These results show that spore adhesion is largely reversible under the assay conditions.

As a function of spore density in the assay, the percentage of total spores which bound was constant over a wide spore density range (1×10^5 to 3×10^6 ml^{-1}) and began to decrease slightly at higher densities. Therefore, in the standard assay (3×10^5 spores ml^{-1}), the hypocotyl area available for binding was not limiting. Light microscope examination of the spores after staining with lactophenol blue showed that very few spores attached to the cut ends of the hypocotyls and that most of the spores were aligned lengthwise on the hypocotyl surface at the junctions of the anticlinal walls of the epidermal cells. A similar orientation of spores was observed when drops of spore suspension were applied to hypocotyls by using the assay described by Bailey and Deverall (2) for testing pathogenicity.

The removal of surface wax from hypocotyls by $CHCl_3$ treatment reduced the number of spores which bound from $2.47 (\pm 0.55) \times 10^5$ to $0.43 (\pm 0.28) \times 10^5$ (these values are the means and standard deviations from five replicates).

Adhesion to microtiter plate wells. In the microtiter plate assay with 10 mM citrate-phosphate buffer, pH 5.0, 30 to 40% of the spores remained bound when the wells were washed after a 1-h incubation (the time required for all spores to settle onto the well surface). When the incubation time was increased beyond 1 h, the number of spores remaining bound after washing decreased (data not shown), possibly due to O_2 exhaustion in the wells since respiration appears to be important for adhesion (see Table 2).

Effect of salts on adhesion. The addition of NaCl to the assay mixtures enhanced spore adhesion to both hypocotyls and polystyrene as compared with the spore adhesion of control mixtures containing deionized water alone (Table 1). Results very similar to those shown for NaCl were obtained when K^+, Ca^{2+}, or Mg^{2+} chloride was used (data not shown). In comparing the effect of salts on adhesion in the two assays, it is important that after a 1-h incubation the salt concentration in the hypocotyl assay with deionized water was equivalent to 4.5×10^{-4} M NaCl (based on conductivity measurements) due to ion leakage. This salt concentration may explain why only about 3% of the spores bound to polystyrene in deionized water, whereas 25 to 35% bound to hypocotyls, and why added salts enhanced adhesion to a

TABLE 1. Effect of NaCl on adhesion of *C. lindemuthianum* spores to *P. vulgaris* hypocotyls and to polystyrene

NaCl concn (M)	Adhesion[a] to:	
	Hypocotyls[b]	Polystyrene[b]
0	2.88 ± 0.31	203 ± 78
10^{-4}	3.17 ± 0.74	374 ± 131
10^{-3}	4.67 ± 0.89	1,693 ± 352
10^{-2}	4.97 ± 0.98	2,176 ± 122

[a] Numbers are means and standard deviations from five replicates and are given as spores bound $\times 10^5$ and spores bound per square millimeter in the hypocotyl and polystyrene binding assays, respectively.

[b] Assays performed in deionized water.

TABLE 2. Effect of respiratory inhibitors on adhesion of *C. lindemuthianum* spores to *P. vulgaris* hypocotyls and to polystyrene

Inhibitor (M)	Adhesion[a] to:	
	Hypocotyls[b]	Polystyrene[c]
None	2.61 ± 0.28	2,215 ± 271
NaN_3 (10^{-4})	0.72 ± 0.40	460 ± 153
Antimycin A (10^{-5})[d]	0.78 ± 0.51	405 ± 135

[a] Numbers are means and standard deviations from five replicates and are given as spores bound × 10^5 and spores bound per square millimeter in the hypocotyl and polystyrene assays, respectively.
[b] Assay performed in deionized water.
[c] Assay performed in 10 mM citrate-phosphate buffer, pH 5.0.
[d] Antimycin A was added as a 10^{-3} M solution in ethanol. Adhesion was unaffected by ethanol alone (1%, vol/vol).

much greater degree in the microtiter plate assay than in the hypocotyl assay.

Effect of respiratory inhibitors. The respiratory inhibitors sodium azide and antimycin A strongly inhibited spore adhesion to hypocotyls and to polystyrene (Table 2).

Effect of proteins and Tween 80. Various commercially available proteins or glycoproteins and the detergent Tween 80 were tested for their effect on adhesion with 10 mM citrate-phosphate buffer, pH 5.0, in the assay mixtures (Table 3). Spore adhesion to both hypocotyls and polystyrene was strongly inhibited by these substances. Proteins had to be added at a higher concentration in the hypocotyl assay than in the microtiter plate assay to produce a similar effect.

DISCUSSION

The similarities between spore adhesion to hypocotyls and spore adhesion to polystyrene in terms of inhibition by respiratory inhibitors, proteins or glycoproteins, and Tween 80 and enhancement by salts strongly suggest that the mechanism of adhesion to both surfaces is the same. Since the plant surface and polystyrene are chemically different, adhesion must be based on purely physical surface properties rather than on group-specific binding sites. Spores were subjected to a constant shear force in the hypocotyl assay but were allowed to settle undisturbed onto the well surface in the microtiter plate assay; it therefore seems likely that spore adhesion to the plant surface under field conditions would show similar characteristics to those reported here even if the physical forces exerted on the spores differ somewhat from those used in the hypocotyl assay.

The ability of respiratory inhibitors to inhibit adhesion indicates a requirement for metabolic activity, which might be necessary for the secretion or synthesis of adhesive material at the spore surface.

The inhibition of adhesion by different proteins presumably results from nonspecific interactions with one or both of the adhering surfaces. Proteins may bind directly to the surface groups involved in adhesion, or proteins may act by preventing close contact between the adhesive surfaces due to their interactions with other surface molecules. Adsorbed monolayers of organic molecules are known to alter the adhesiveness of solid surfaces (1), and protein adsorption to polystyrene has been reported to inhibit bacterial attachment (10). Proteins also bind to spores and hyphal cell wall fragments by means of weak nonspecific interactions, as demonstrated previously by their ability to cause agglutination under appropriate conditions (25). Similar interactions of proteins with the spore surface may contribute to the inhibition of adhesion in the present study, although it should be noted that the present results are not directly comparable with the earlier study in which very gentle shaking was important for agglutination and the buffers used contained azide, which suppressed spore adhesion to hypocotyls and microtiter plate wells in the present work. Spore agglutination by proteins was not observed in the assay of spore adhesion to hypocotyls, possibly due to the shear force applied in the assay. In the microtiter plate assay, only a very slight aggregation of spores occurred, perhaps because of a low frequency of contact between spores during settling, and adhesion was more sensitive to inhibition by proteins than in the hypocotyl assay. This latter result may have been due to the different physical forces exerted on the spores in the two assays. Alternatively, a stronger affinity of proteins for polystyrene than for the plant surface might be responsible for the greater inhibition.

Cations have been reported to promote adhesion or agglutination in a number of systems. Such effects have been explained by the ability of multivalent cations to bridge, or neutralize, two negatively charged surfaces (6, 16) or in terms of the effect of electrolytes on the thickness of electrical double layers between the adhering surfaces (15, 23), based on the DLVO theory of the stability of colloids (7, 22). These explanations are unsatisfactory in the present case, however, since divalent ions should be much more

TABLE 3. Effect of proteins and Tween 80 on adhesion of *C. lindemuthianum* spores to *P. vulgaris* hypocotyls and to polystyrene

Test substance	Spores bound[a] at the given concn of test substance			
	Hypocotyls[b]		Polystyrene[b]	
	10 μg/ml	100 μg/ml	1 μg/ml	10 μg/ml
None	—[c]	—[c]	—[c]	—[c]
Cytochrome *c*	2.17 ± 0.24	0.92 ± 0.51	86 ± 40	8 ± 16
Hemoglobin	1.22 ± 0.26	0.42 ± 0.24	234 ± 97	70 ± 118
Ovalbumin	1.62 ± 0.78	0.98 ± 0.47	757 ± 196	257 ± 152
Bovine serum albumin	1.11 ± 0.54	0.00 ± 0.59	803 ± 215	55 ± 68
Fetuin	0.91 ± 0.32	0.12 ± 0.14	1,474 ± 274	78 ± 40
Immunoglobulin	0.84 ± 0.14	0.00 ± 0.17	1,739 ± 168	62 ± 44
Tween 80	0.66 ± 0.20	0.51 ± 0.50	2,192 ± 218	179 ± 53

[a] Numbers are means and standard deviations from five replicates and are given as spores bound × 10^5 and spores bound per square millimeter in the hypocotyl and polystyrene assays, respectively.
[b] Assays performed in 10 mM citrate-phosphate buffer, pH 5.0.
[c] —, When no test substance was added, 3.55 ± 0.56 × 10^5 spores bound in the hypocotyl assay, and 2,324 ± 165 spores bound per mm^2 in the polystyrene assay.

effective than monovalent ones in promoting adhesion by such mechanisms (6, 15).

A number of results suggest the involvement of hydrophobic interactions in the adhesion mechanism, and it is possible that salts might promote adhesion by enhancing such interactions between the adhering surfaces. Hydrophobic bond formation depends mainly on the gain in entropy obtained because of a decrease in the contact of hydrophobic groups with water, and ions enhance the association of hydrophobic groups by their effect on the structure of water (21). Such effects are poorly understood, but most solutes appear to show a rather similar effect in increasing the strength of hydrophobic interactions (3). The plant surface probably exhibits many hydrophobic groups due to the presence of surface wax, and it may be significant that spores adhered less well to hypocotyls after the removal of surface wax. It is also likely that the adhesion of spores to polystyrene depends on hydrophobic interactions; studies with bacteria have demonstrated a strong correlation between cell surface hydrophobicity and the ability to adhere to polystyrene (19). A further indication that hydrophobic bonding could be involved in spore adhesion is the ability of Tween 80 to reduce adhesion, since this wetting agent would be expected to render hydrophobic surfaces more hydrophilic. This detergent also prevents the orientation of certain bacteria at oil-water interfaces and their adsorption to solid surfaces, phenomena which appear to result from hydrophobic interactions (14).

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